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A Functional Marker Centromere with No Detectable Alpha-Satellite, Satellite III, or CENP-B Protein: Activation of a Latent Centromere?

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Summary

We report the investigation of an unusual human supernumerary marker chromosome 10 designated "mar del(10)." This marker is present together with two other marker chromosomes in the karyotype of a boy with mild developmental delay. It has a functional centromere at a primary constriction and is mitotically stable. Fluorescence in situ hybridization (FISH) using alpha-satellite and satellite III DNA as probes failed to detect any signal at the primary constriction site. CENP-B protein could not be demonstrated, although the presence of at least some centromeric proteins was confirmed using a CREST antiserum. Consideration of these and other cytogenetic and FISH results supports a mechanism of formation of the mar del(10) chromosome involving the activation of a latent intercalary centromere at 10q25.

Introduction

In eukaryotic organisms, proper mitotic and meiotic chromosomal segregation is dependent on the centromere. After replication, the centromere holds the sister chromatids together until metaphase and interacts with the spindle fibers to effect chromosome separation during anaphase. At present, it is not clear how these functions are carried out at the molecular level.

The centromere of human metaphase chromosomes appears as a primary constriction, which is heterochromatic in nature. It is made up of a large amount of highly repeated satellite DNA. Alpha-satellite is the best characterized of these DNA. It is composed of tandemly repeated monomeric units of 171 bp and constitutes 3%–5% of each chromosome. A characteristic of this DNA is the evolution of distinct subfamilies that are specific to one or a small group of the human chromosomes (reviewed in Choo et al. 1991). Alpha-satellite DNA is believed to be important for centromeric function, because of its presence at all human centromeres (Manuelidis 1978; Mitchell et al. 1985; Choo et al. 1991) and because it is known to bind at least two centromere proteins: CENP-B (Masumoto et al. 1989) and an HMG-like nuclear protein (Strauss and Varshavsky 1984). This DNA has also been implicated in the formation of a functional centromere after its transfection into cultured monkey cells (Haaf et al. 1992).

Besides alpha-satellite DNA, the pericentric regions of human chromosomes are known to contain classical satellite DNA I–III (Prosser et al. 1986). As determined by in situ hybridization, these sequences are present in greatly varying amounts, ranging from very high levels on some chromosomes to low or undetectable levels on other chromosomes (Beridze 1986, pp. 30–96; Choo et al. 1990*a*, 1992; Grady et al. 1992; Kalitsis et al., in press). Satellite III DNA is of particular interest: because of its observed evolutionary conservation, centromeric location, unusual hydrogen bonding properties, and high affinity for specific nuclear proteins, it has been proposed to be a possible candidate for the kinetochore DNA (Grady et al. 1992).

A third putative centromeric DNA is a unique or low-copy sequence known as "CM8." This DNA is

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considered an essential component because it is detected at all human centromeres and because it forms an active centromere when transfected into cultured mouse cells (Hadlaczky et al. 1991). However, more recent studies have mapped this sequence at 9qter and suggested that the sequence cannot be a component of a normal centromere (Cooper and Tyler-Smith 1992; McGill et al. 1992).

During the course of routine diagnostic cytogenetic analysis, we studied a patient with developmental delay in whom a stable chromosomal marker with little or no centromeric heterochromatic material was detected. We describe here detailed cytogenetic and molecular characterization of this marker and present evidence in support of the possible existence of latent intercalary centromeres within the human genome.

Material and Methods

Cytogenetics Studies

Metaphase chromosomes were prepared from cultured peripheral blood lymphocytes, Epstein-Barr virus–transformed lymphoblast cell lines, or skin fibroblasts. Chromosomes were analyzed by GTL-, CBG- and AgNOR-banding and plain staining (Rooney and Czepulkowski 1986).

In Situ Hybridization

Fluorescence in situ hybridization (FISH) was carried out according to established methods (Pinkel et al. 1986). DNA probes were labeled with biotin-16-dUTP by using random priming (Boehringer). Hybridization was performed at 37°C in 50% formamide, 2 × SSC and were washed at 42°C in 50% formamide, 1 × SSC. For low-stringency conditions, the same solutions were used, but hybridization and washings were both carried out at ambient temperature.

Detection of probe was made using fluorescein isothiocyanate (FITC)–conjugated avidin. Where indicated, up to three rounds of amplification of the hybridization signals were performed by incorporating multiple "sandwich" layers of biotinylated goat anti-avidin and avidin-FITC. Slides were mounted in glycerol containing 100 mg/ml 1,4-diazabicyclo[2,2,2]-octane (DABCO) diluted 2:1 with 0.1 M HCO_3^- (pH 8.6). Propidium iodide and DAPI at final concentrations of 10 μg/ml and 2.5 μg/ml, respectively, were included in the mountant to allow identification of the chromosomes. The chromosomes showed plain red staining under blue light and showed a G-banding pattern under UV light that allowed chromosome identification. Chromosomes were photographed using ASA 400 Kodak Ektachrome EES slide film, followed by E6 processing. Selected photographs were reproduced by Cibachrome method into color prints or were printed directly onto black-and-white paper.

Chromosome-specific, centromeric alpha-satellite probes used for FISH analysis included alphaXT (specific for chromosomes 14 and 22; Jorgenson et al. 1988), alphaRI (specific for chromosomes 13 and 21; Jorgenson et al. 1987), pTRA-25 (specific for chromosome 15; Choo et al. 1990*b*), D10Z1 (specific for chromosome 10; Oncor), TRX (specific for X chromosome; Choo et al. 1987), and Y84 (specific for Y chromosome; Wolfe et al. 1985). These probes were used individually at a concentration of 25 ng/15 μl of hybridization buffer, under a 4.4 cm^2 coverslip, at 37°C.

For the detection of all alpha-satellite DNA within the genome, a mixture of alpha-satellite probes including alphaXT, alphaRI, pTRA-25, TRX, and Y84 was prepared. This mixture was used at a concentration of 50 ng/15 μl of hybridization buffer under low stringency. For the detection of human satellite III DNA, a Y chromosome–derived pY3.4 probe (Lau et al. 1985) was used, again under low stringency.

In addition to the above probes, a chromosome 10–specific paint (Oncor) hybridizing to unique sequences spanning the length of the chromosome was employed for the determination of the origin of the marker chromosomes. Chromosome in situ suppression hybridization was performed according to the manufacturer's recommended procedure.

Centromeric Antibodies

Metaphase chromosomes from fibroblast cultures in aqueous suspension were spread onto slides by using Cytospin and were fixed for 15 min in 80% ethanol at 4°C. Human centromeric protein was detected by indirect immunofluorescence using two different antisera. The first (designated "CREST #3" and provided to us by S. Wittingham) was derived from a patient with the CREST form of autoimmune scleroderma. In western blot analysis against purified human centromere proteins CENP-A, CENP-B, and CENP-C, this antiserum was shown to be specific for CENP-A only (G. McColl, personal communication). For metaphase chromosome studies, binding of the primary antibody was detected with FITC-conjugated mouse anti-human IgG (Jackson Laboratory).

The second antiserum (designated "Rab-L" and provided to us by W. C. Earnshaw) was raised in rabbit, against cloned CENP-B expressed in *Escherichia coli*. Detection of the primary antibody on chromosome

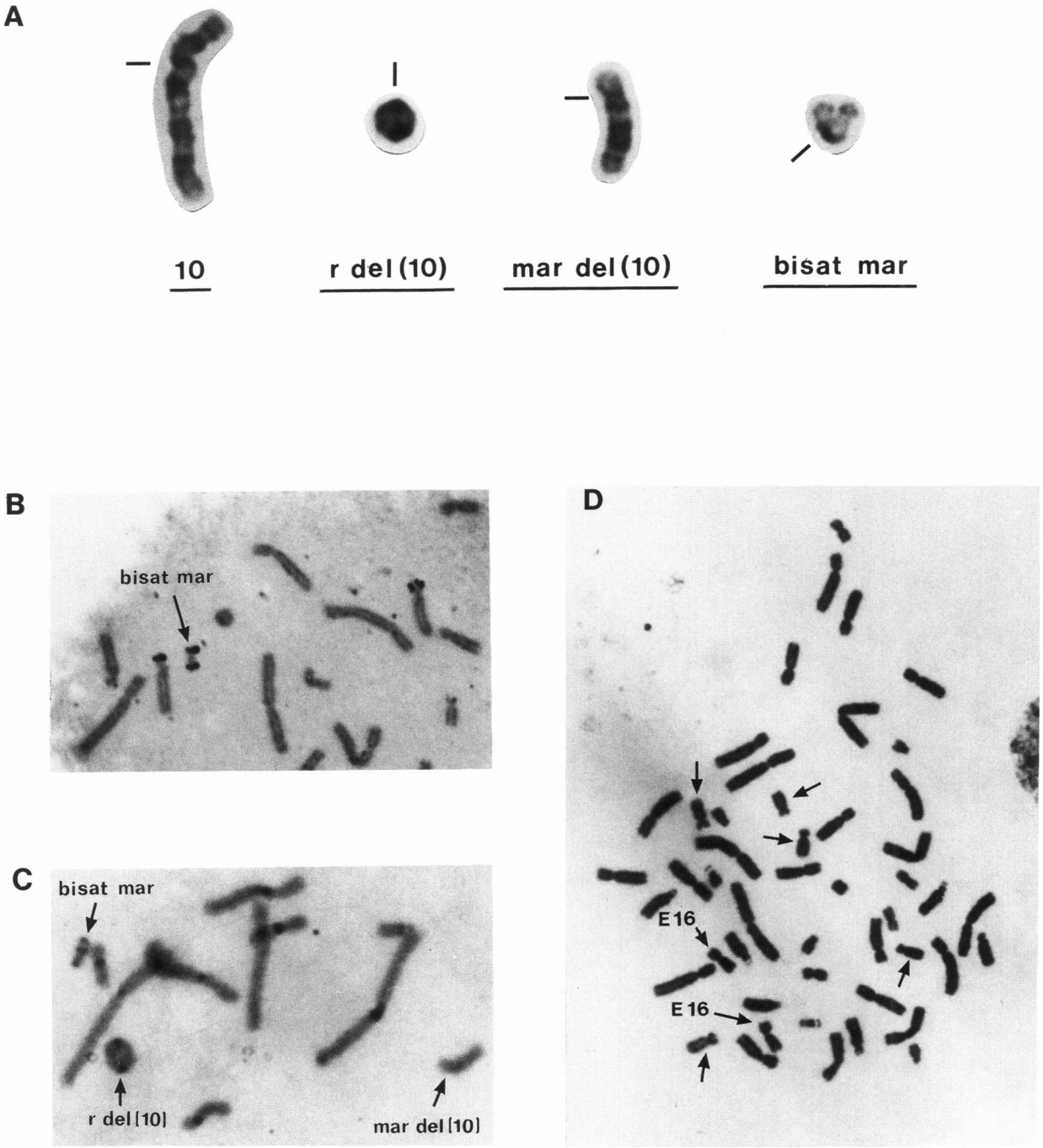
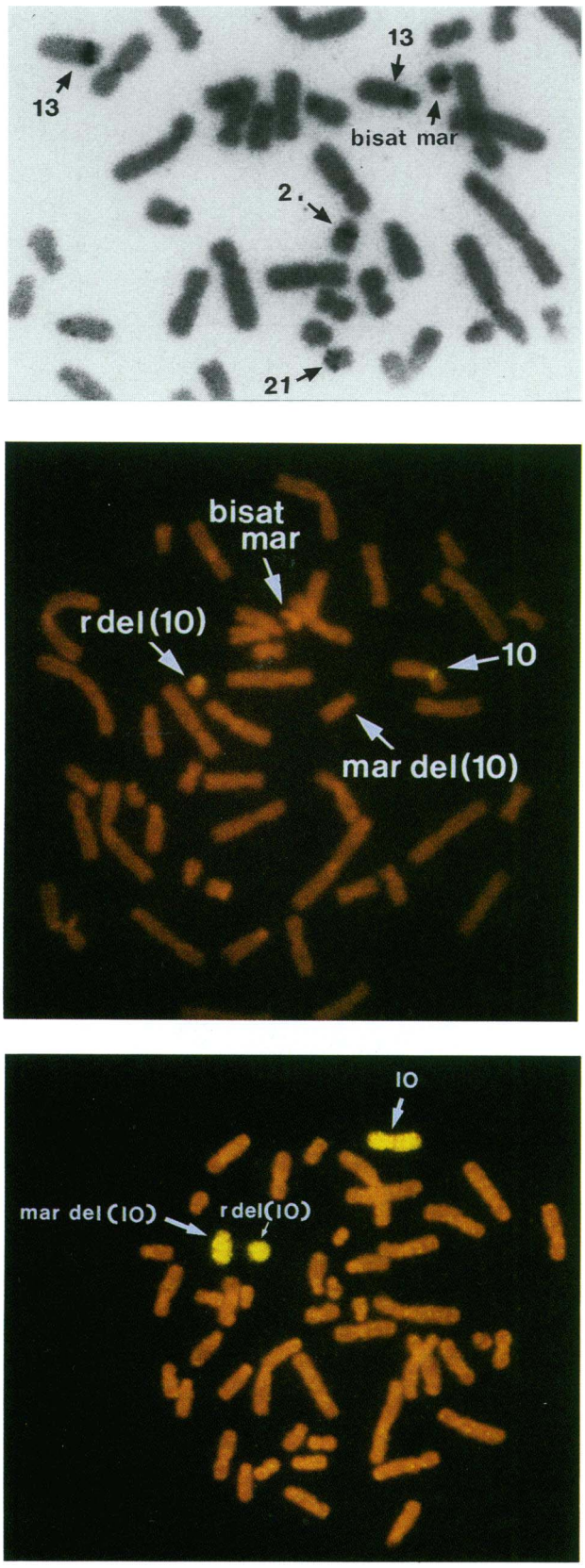


Figure 1 Cytogenetic analysis of proband's chromosomes. *A*, Partial karyotype showing GTL-banded normal chromosome 10, r del(10), mar del(10), and the bisatellited marker (bisat mar). Positions of centromeres are indicated. *B*, AgNOR-banding showing two active ribosomal gene clusters on the bisatellited marker (bisat mar). *C*, CBG-banding showing positive signals on a double-ring r del(10) chromosome (refer to text), the bisatellited marker (bisat mar), and other chromosomes. Note the negative staining for the mar del(10) chromosome. *D*, Plain staining showing the presence of a primary constriction in all the E-group chromosomes (*arrows*) including mar del(10), although the latter cannot be readily distinguished from the other E-group chromosomes by this staining. The two chromosomes 16 are indicated.



slides was achieved with DTAF-conjugated donkey anti-rabbit IgG (Jackson Laboratory).

Results

Clinical Features

The proband was referred for diagnostic chromosome analysis because of developmental delay. When examined at 4 years 10 mo, he had delayed speech development but normal motor activity and was not dysmorphic. His height was 106.5 cm (>25th centile), weight was 16.8 kg (25th centile), and head circumference was 49 cm (25th centile).

Cytogenetic Studies

GTL-banding analysis of metaphase cells from the patient demonstrated the presence of three marker chromosomes (fig. 1*A*) and the absence of a normal chromosome 10 in an otherwise normal karyotype. One of the markers was a monocentric, bisatellited chromosome (fig. 1*B*). On the basis of the G-banding pattern, it appears that the other two markers were both derived from chromosome 10. One of these formed a ring and is designated "r del(10)," while the other represented a deleted chromosome 10 designated "mar del(10)." The karyotype of the patient can therefore be described as 48,XY,-10,+r del(10)(p12.2 →q-23.3),+mar del(10)(pter → p12.2::q23.3 → qter),+bisatellited marker. Further support for this interpretation was obtained from in situ hybridization studies.

Karyotype analysis of the proband's phenotypically normal parents revealed a normal karyotype in the mother, whereas the father carried the same bisatellited marker seen in the proband but was otherwise normal. This bisatellited marker chromosome was also present in the proband's phenotypically normal sister.

The stability of the marker chromosomes was investigated by examining their segregation in dividing cultured fibroblast and lymphocyte cells derived from the patient. More than 100 cells were analyzed for each of the two tissue types. The mar del(10) and bisatellited markers were present in every cell examined, suggesting complete stability of these chromosomes in mitotic segregation. With the r del(10) marker, anomalies includ-

Figure 2 FISH analysis of proband's chromosomes, using the following as probes: (*top*) alphaRI, an alphoid probe specific for the centromeres of chromosomes 13 and 21; (*middle*) D10Z1, an alphoid probe specific for the centromere of chromosome 10; and (*bottom*) a chromosome 10–specific paint. Chromosomes were counterstained with propidium iodide.

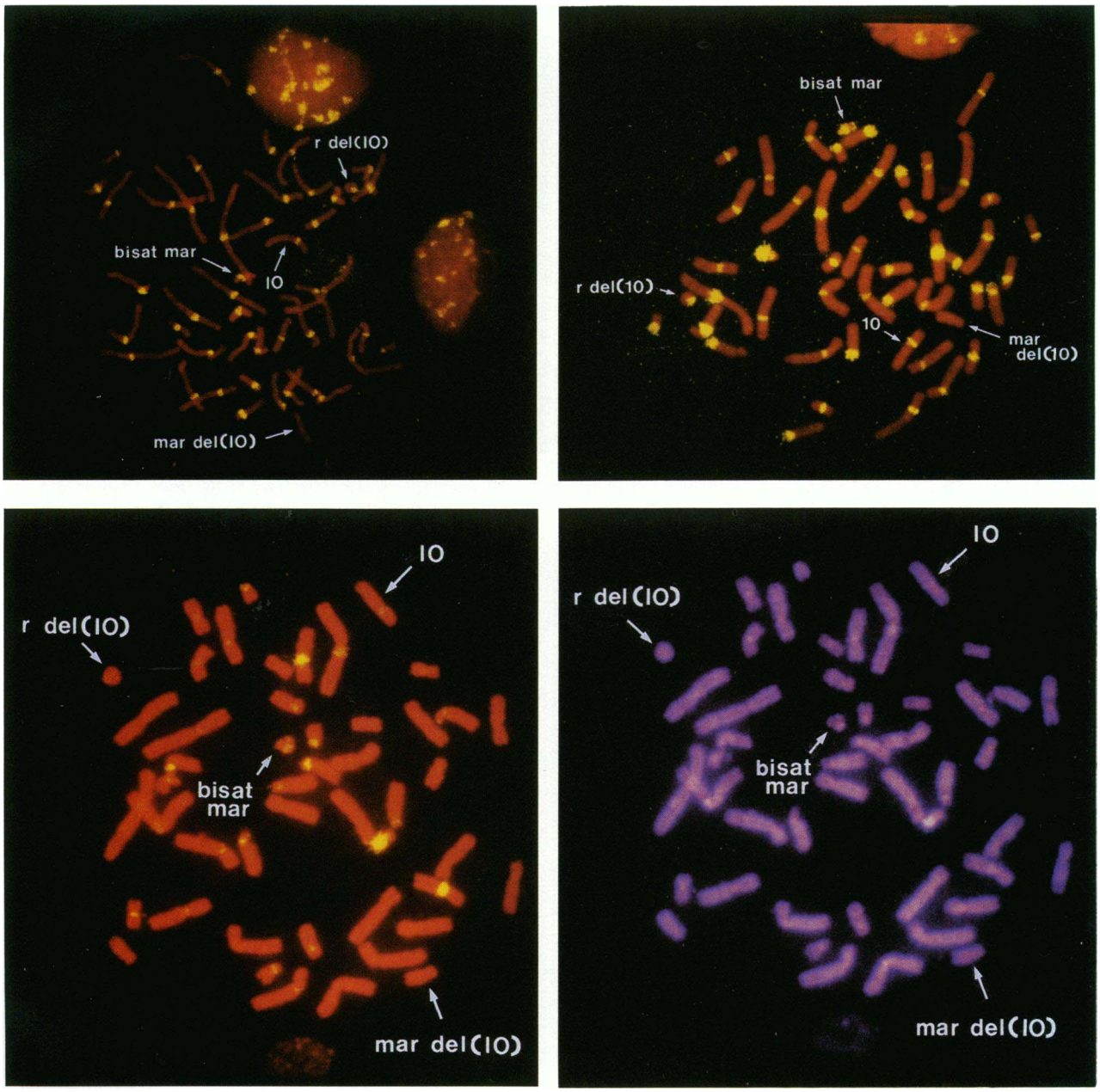


Figure 3 FISH analysis of proband's chromosomes, using the following as probes: (*top left*) pooled alpha-satellite sequences; a satellite III DNA stained with (*bottom left*) propidium iodide and (*bottom right*) DAPI (both stains are of the same cell); and (*top right*) a mixture of alphoid and satellite III sequences. Hybridization was at low stringency.

ing loss of chromosome and the appearance of double rings (see fig. 1C) were found in 4%–8% of the cells.

CBG-banding showed positive staining of r del(10) and the bisatellited marker (fig. 1C). The mar del(10) chromosome, on the other hand, was C-band negative, suggesting the presence of little or no centromeric heterochromatin. On plain staining, a primary constriction was seen on all the chromosomes, including the mar del(10) (fig. 1*D*).

In Situ Hybridization

FISH was employed to characterize the three marker chromosomes. Hybridization of the bisatellited marker with alphaRI, alphaXT, and pTRA-25 under high strin-

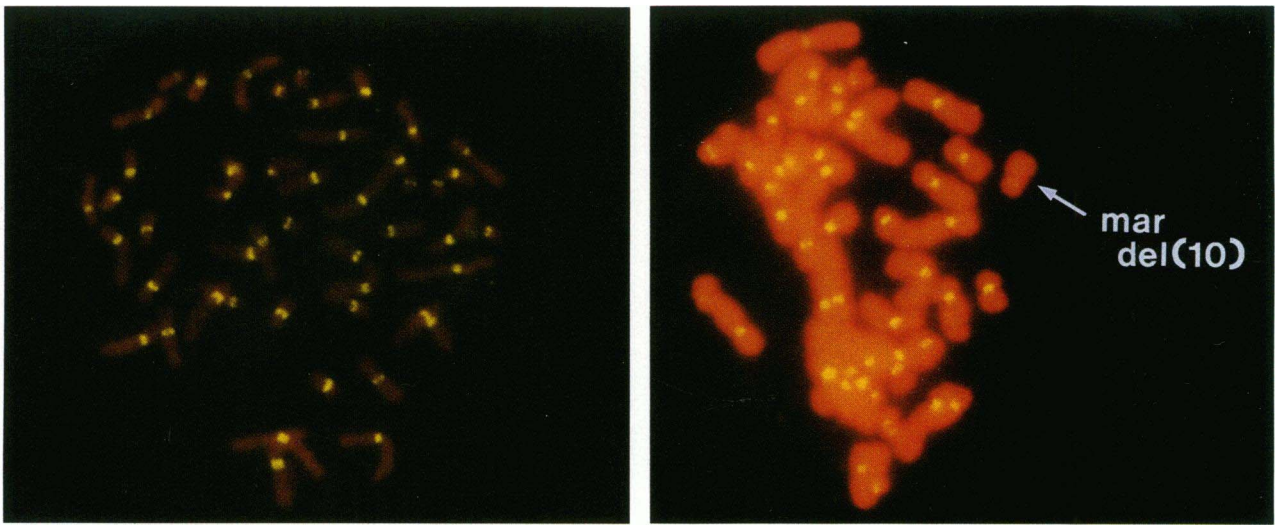


Figure 4 Detection of centromere proteins on proband's chromosomes, using CREST #3 antiserum (the two panels present material from two different cells). Cells were counterstained with propidium iodide.

gency gave a positive signal with the alphaRI probe only, indicating its origin from chromosome 13 and/or chromosome 21 (fig. 2, *top*). When an alphoid probe specific for the centromere of chromosome 10 was used, a positive signal was detected on the normal chromosome 10 and the r del(10) marker but not on the mar del(10) chromosome (fig. 2, *middle*). Finally, FISH analysis using a noncentromeric, chromosome 10–specific paint established that both r del(10) and mar del(10) were derived from chromosome 10 and that no detectable amount of this chromosomal material has been translocated onto any other chromosome (fig. 2, *bottom*).

The mar del(10) chromosome was further investigated using a mixture of five different alphoid DNA probes under low-stringency FISH conditions (see Material and Methods). In more than 50 cells examined, no hybridization was seen on this marker chromosome, even after multiple rounds of amplification with biotinylated goat anti-avidin and avidin-FITC to enhance the signal, whereas a positive hybridization was seen in the centromeric regions of all other chromosomes (fig. 3, *top left*).

In situ hybridization using a satellite III probe at low stringency gave signals on 34/48 of the centromeres, including the bisatellited marker, the normal chromosome 10, and the r del(10) marker. No hybridization was detectable on the mar del(10) chromosome (fig. 3, *bottom left* and *bottom right*). The failure to observe a signal on a chromosome by using this probe was likely due to the complete absence—or the presence of subdetectable levels—of satellite III–related DNA on specific chromosomes, rather than to inefficiency of hybridization, since the hybridization pattern was reproducible in different cells.

The top-right panel of figure 3 shows results of hybridization using a combination of pooled alpha-satellite and satellite III probes under low stringency and after saturating rounds of amplification of the FITC signals. The results confirmed those seen in the top-left, bottom-left, and bottom-right panels of figure 3, in demonstrating no signal on the centromere of the mar del(10) chromosome.

Centromeric Antibodies

Since identification of individual chromosomes is rarely possible with the preparations obtained using the aqueous spreading necessary for the detection of centromeric proteins, a combination of DAPI staining, chromosome counting, and chromosome size estimation was used for data analysis. The CREST #3 antiserum was used in the following studies. In metaphase spreads where the chromosomes were well separated, a total of 48 centromeric signals were counted and corresponded to the 48 chromosomes found in the proband's karyotype (fig. 4, *left*). In the right panel of figure 4, while the separation and morphology of most of the chromosomes were poor, the mar del(10) chromosome was discernible by DAPI staining (not shown) and was demonstrated to be positive with the antibody. Note that the signal on the mar del(10) chromosome appeared to be decreased compared with those of most of

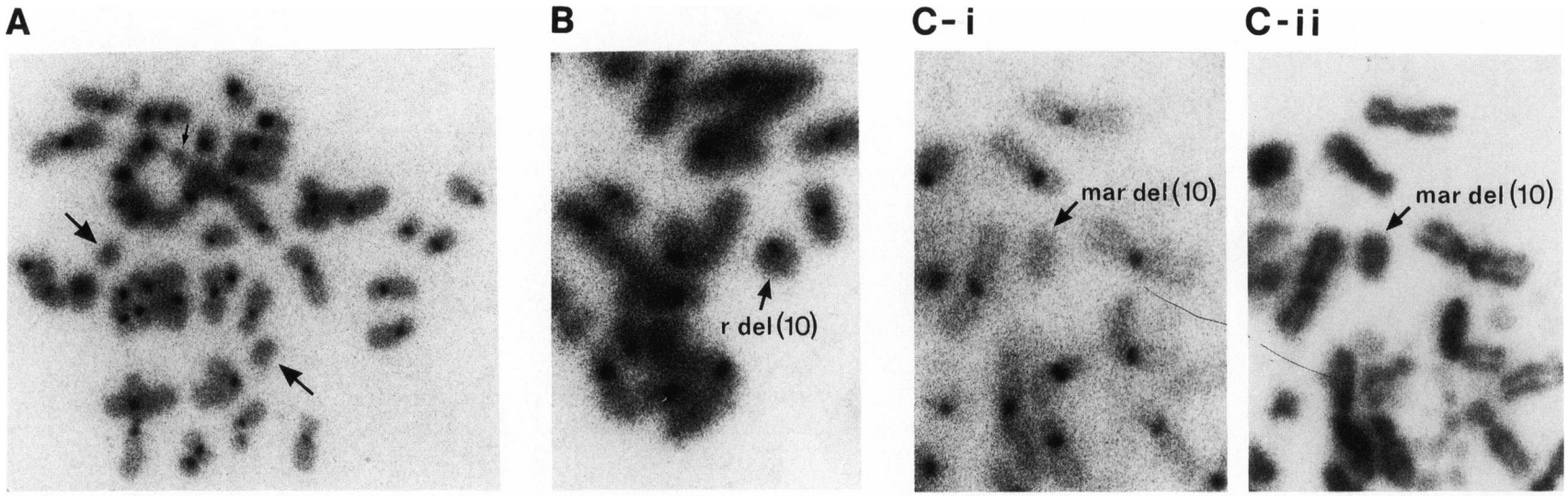


Figure 5 Detection of centromere proteins on proband's chromosomes, using anti–CENP-B antibodies. *A*, Complete metaphase showing 45 positive chromosomes and 3 chromosomes with little or no detectable signal. The smaller arrow indicates the bisatellited marker, whereas the larger arrows indicate mar del(10) and the Y chromosome, which cannot be confidently distinguished on the basis of DAPI staining of the present cell (see *B* and *C* and text). *B*, Partial metaphase showing positive CENP-B signal on r del(10). *C-i* and *C-ii*, Partial metaphase showing negative antibody staining on mar del(10), when the same cell is counterstained with propidium iodide (*C-i*) or DAPI (*C-ii*).

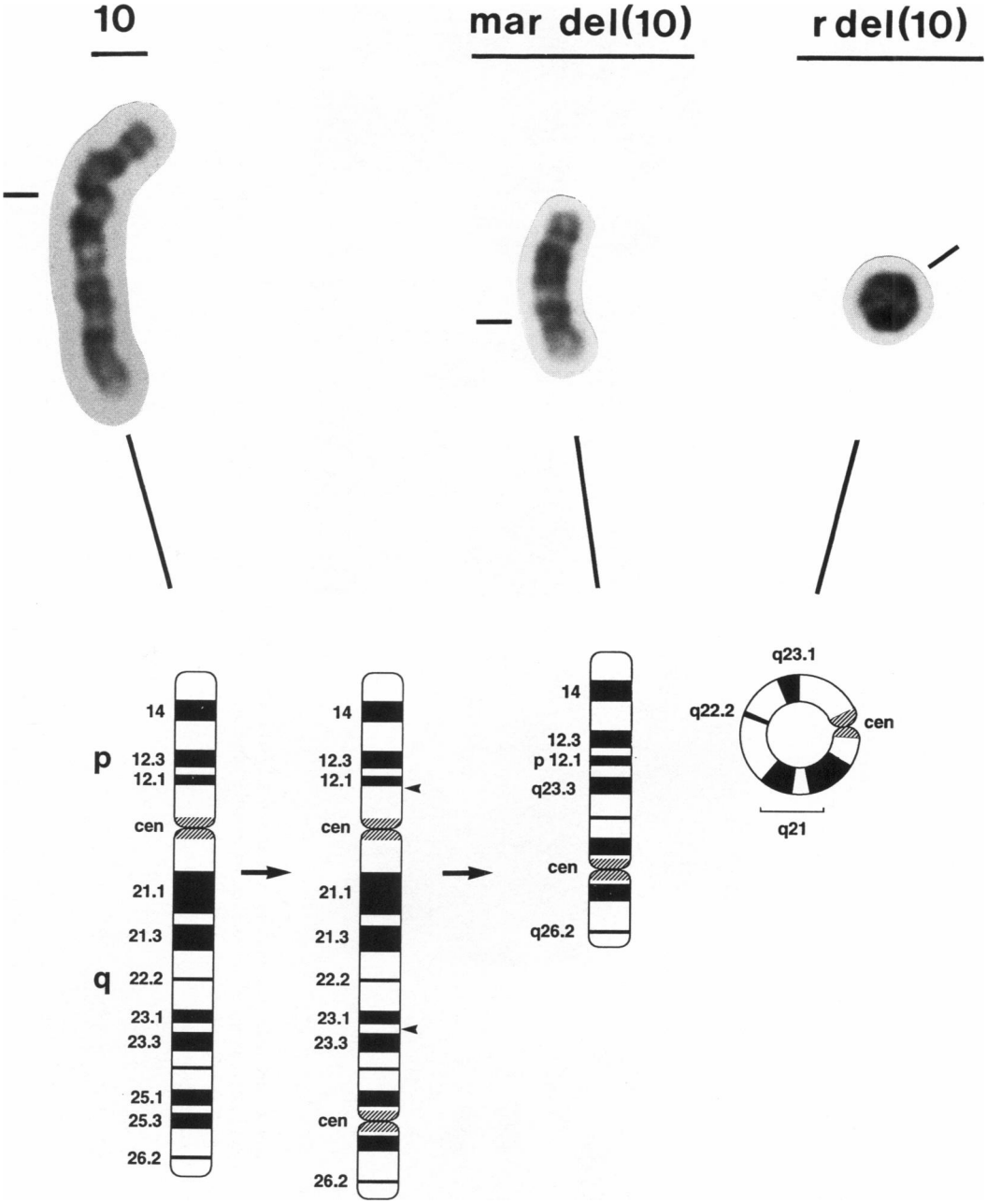


Figure 6 Mechanism for the formation of the mar del(10) and r del(10) chromosomes. The first step involves the generation of a new centromere at q25, followed by two breaks (*arrowheads*) and rejoining. GTL-banded metaphase chromosomes corresponding to the normal chromosome 10, mar del(10) and r del(10), are shown, with the positions of centromeres indicated.

the other chromosomes. The significance of these results will be discussed below.

When the proband's chromosomes were investigated using the anti-CENP-B antiserum, strong centromere signals were consistently detected in 45 of the 48 chromosomes (fig. 5*A*). The r del(10) chromosome was positive with the antibody (fig. 5*B*). Although the bisatellited marker was one of the three "negative" chromosomes, it showed very weak, but discernible, signal under direct microscopic examination, thereby indicating the presence of a small amount of CENP-B protein (not shown). The remaining two negative chro-

mosomes were identified as the mar del(10) marker and the Y chromosome, on the basis of a combination of DAPI staining (fig. 5C), size of the chromosomes, and knowledge that the human Y chromosome has no reactivity with antibodies against CENP-B protein (Pluta et al. 1990).

Discussion

A patient with developmental delay was found to have an unusual karyotype with a missing chromosome 10 and three marker chromosomes. One of the markers is a paternally inherited, monocentric, bisatellited chromosome that is likely to have originated from chromosome 13 and/or chromosome 21. On the basis of cytogenetic banding and FISH analyses, we established that the other two markers, mar del(10) and r del(10), were derived from chromosome 10. For the following reasons, we infer that there was little or no loss of chromosome 10 material in the formation of these two markers: (i) the patient's clinical presentation suggests that he is not monosomic for large regions of chromosome 10; (ii) the aggregate size of the two chromosomes amounts approximately to a normal chromosome 10; (iii) the G-banding patterns agree well with a rearranged chromosome 10 (see mechanism below); and (iv) there is no cytogenetic or FISH evidence to suggest either the translocation of chromosome 10 materials onto other chromosomes or the involvement of other chromosomes in the formation of these two markers.

The observation of a primary constriction and the ability of the mar del(10) chromosome to segregate stably both in vitro and in vivo indicate the presence of a functional centromere in this marker. It is therefore of interest that this chromosome contains little or no centromeric heterochromatin, as is evident from both negative CBG-banding and the lack of detectable alphoid and satellite III DNA. These results suggest either that alpha-satellite and satellite III are not essential for full centromeric activity or that only a relatively small amount of these DNAs (beyond the sensitivity of our in situ hybridization detection) is sufficient.

We used centromeric antibodies to look for the presence of centromeric protein on the mar del(10) chromosome. A positive signal with the CREST #3 antiserum indicated that at least some centromeric proteins are present at the primary constriction, although the observation of a decreased signal (fig. 4, *right*) suggests a reduction in the amount of one or more of these proteins. Although the CREST #3 antiserum has previously been tested and shown to contain antibodies against CENP-A but not against CENP-B and CENP-C, the presence of antibodies against other centromeric proteins has not been excluded. As such, it is not possible at this stage to designate the protein(s) that we are detecting with this antiserum.

When the mar del(10) chromosome was further examined with an antiserum specific for the CENP-B protein, no detectable signal was seen. This indicates that very little or no CENP-B protein exists on the centromere of this marker chromosome. The detection of a greatly reduced CENP-B signal on the bisatellited marker is puzzling, given that we observed an abundance of alphoid DNA on this chromosome (fig. 2, *top*). One possible explanation for this is, as has been proposed for the Y chromosome (Pluta et al. 1992), that the bulk of the alphoid DNA on the bisatellited chromosome contains a CENP-B box homologue that was not detectable with the antiserum. Since the centromere of this marker chromosome has originated from chromosome 13 and/or chromosome 21, which bind positively to anti-CENP-B antibodies, it would appear that there has been either a preferential loss of sequences containing the wider consensus CENP-B box or a suppression of CENP-B protein binding.

We considered two possible mechanisms that may result in the formation of the mar del(10) and r del(10) chromosomes. The first involves two breaks, with one occurring within the centromere, followed by an inversion. This would give rise to a dicentric chromosome. As dicentric chromosomes are inherently unstable, subsequent breakage and rejoining would lead to the formation of two marker chromosomes. However, a close examination of the GTL-banding patterns of these chromosomes does not support this mechanism.

In the second mechanism (fig. 6), the initial step involves the generation of a centromere within the q25 region of the long arm of chromosome 10. After the formation of this centromere, the unstable dicentric chromosome breaks and rejoins to form the mar del(10) and r del(10) chromosomes. This mechanism is strongly supported by results of GTL banding and FISH painting of the two marker chromosomes (figs. 1*A* and 2, *middle* and *bottom*). However, it is necessary to explain the origin of the supernumerary centromere within band q25. Two possible schemes may be proposed in this regard. The first requires the breakage of part of a normal centromere, followed by its insertion into band q25 of chromosome 10. However, because of the lack of evidence for either the presence of normal centromeric material on mar del(10) or the disruption of a normal centromere, we consider this scheme to be unlikely.

The second scheme requires the generation of a cen-

tromere in situ by the activation of a latent centromere. The concept of the existence of "latent intercalary centromeres" has been postulated by a number of workers to explain the evolution of primate chromosomes (see Dutrillaux 1979). The detection of low levels of alphoid DNA at noncentromeric 2q21 and distal 9q13 regions has also been interpreted as evidence for remnants of ancestral centromeres (Aleixandre et al. 1987; Baldini et al. 1991). Further, in studies involving pseudodicentric chromosomes, it has been documented that in rare cases the inactive centromere can become active (Dewald et al. 1979). Although there is no evidence that 10q25 has been the site of an ancestral centromere in recent primate evolution (Yunis and Prakash 1982), on the basis of the above considerations and our cytogenetic and molecular analyses, we consider it possible that one of the key steps in the derivation of the mar del(10) chromosome—and of the corresponding r del(10)chromosome—is the activation of a preexisting, latent intercalary centromere within band 10q25. If this proves to be the case, then the molecular composition of the latent centromere may be quite different from that of a normal centromere, as results of the present study indicate. It is also possible that this phenomenon is not unique to this case but may explain the origin of the centromere in a number of other stable markers derived from various chromosomes that have recently been shown to be alphoid and/or satellite III negative (Crolla et al. 1992; Magnani et al. 1992; Rauch et al. 1992; L. E. Voullaire, unpublished data).

Acknowledgments

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